Expression and activity of mitogen activated protein kinases in human colorectal carcinoma

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Abstract

***Background*—Mitogen activated protein kinases (MAPKs) play a central role in the regulation of both cell growth and differentiation. They are involved in signal transduction of oncogenes and growth factors. The role of MAPK in colonic carcinoma is unknown.**

***Aims*—To establish whether the expression and activity of p42/44 MAPKs are altered in colorectal tumours as compared with normal mucosa.**

***Methods*—The expression and activity of p42/p44 MAPK were investigated in 22 colorectal carcinomas, four adenomas, and the corresponding normal colorectal mucosa by the use of western blotting, immunoprecipitation, and in vitro kinase assays.**

***Results*—After immunoprecipitation with an antibody specific for p42 MAPK, we found significant inactivation of p42 MAPK in colonic carcinomas as well as in adenomas, whereas most sample pairs showed only minor differences in p42 MAPK expression. Investigation of MAPK with an antibody capable of detecting both p42 and p44 MAPK showed a slight but significant decrease in p44 MAPK content in malignant tissues. With this antibody, only minor alterations in MAPK activity and no correlation with p42 MAPK activity were found.**

***Conclusions*—Inactivation of p42 MAPK could be associated with colonic carcinogenesis.**

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Keywords: mitogen activated protein kinase (MAPK); Raf-1; colorectal cancer

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Mitogen activated protein kinases (MAPKs), including the subfamily of extracellular signal regulated kinases, are a family of serine/threonine kinases that are activated in response to various stimulating agents such as growth factors and stress.[1] The 42 kDa MAPK (extracellular signal regulated kinase 2) and 44 kDa MAPK (extracellular signal regulated kinase 1) are known to be components of signalling pathways which influence cellular proliferation and differentiation. These kinases are phosphorylated and activated as a result of the activation of the Ras-Raf-1-MAPK kinase (MEK) pathway. Activated Ras indirectly induces the phosphorylation of Raf-1, which activates MEK by threonine/serine phosphorylation.[2] The dual specific kinase MEK activates p44/42 MAPK by phosphorylation of threonine and tyrosine residues.[3] Activated MAPKs translocate to the nucleus and activate transcription factors such as c-Jun and $p62^{TCF}$ by phosphorylation.[4 5] This regulates transcription of specific genes.

The key role of p42/44 MAPKs in regulating both cellular proliferation and differentiation has been investigated in a multitude of cell culture studies, although only a few papers report the activity and expression of MAPKs in human neoplasms. The study of Oka *et al*[6] showed constitutive activation of p42/44 MAPKs in human renal carcinoma. In the gastrointestinal tract, p42 MAPK has been shown to be activated in chicken crypt cells, but inactivated in villus tip cells.[7] As crypt cells resemble colonic carcinoma cells in some respects, and because activating *ras* mutations are present in up to two thirds of colonic cancers,[8] it was suspected that p42/44 MAPK is also activated in colorectal neoplasms.[7]

In a previous study,[9] however, we found decreased phosphorylation of Raf-1 in colonic carcinomas compared with normal mucosa. This could be due to inactivation of p42/44 MAPKs in the tumours, because p42/44 MAPKs are able to phosphorylate Raf-1.[3] In this study, we have examined the activity and expression of p42/44 MAPKs in colonic cancer and adenomas. Our results show that the activity of p42 MAPK is downregulated in colorectal neoplasms.

Methods

TISSUES

Paired samples of colorectal mucosa and adenocarcinomas derived from 22 patients (44–89 years of age) who were operated on for colorectal carcinoma in the Department of General Surgery, University of Freiburg, were investigated. Six tumours were located in the ascending colon, one in the descending colon, seven in the sigmoid, and eight in the rectum. Nine were classified (tumour, node, metastasis (TNM) classification) as $T_xN_xM_{1-2}$, six as $T_xN_{1-3}M_0$, and seven as $T_xN_0M_0$. One tumour was graded as G1, 16 as G2, five as G3. Four samples derived from colonic adenomas were also studied.

Tumour samples were taken from vital areas of histopathologically confirmed carcinomas and adenomas. Mucosal samples were derived from unaffected mucosa, 2 cm distal to the oral resection margin, by sharp dissection from the submucosa. The tissues were harvested imme-

Abbreviations used in this paper: MAPK, mitogen activated protein kinase; SSU, seastar units; SDS/PAGE, sodium dodecyl sulphate/polyacrylamide gel electrophoresis; MEK, MAPK kinase.

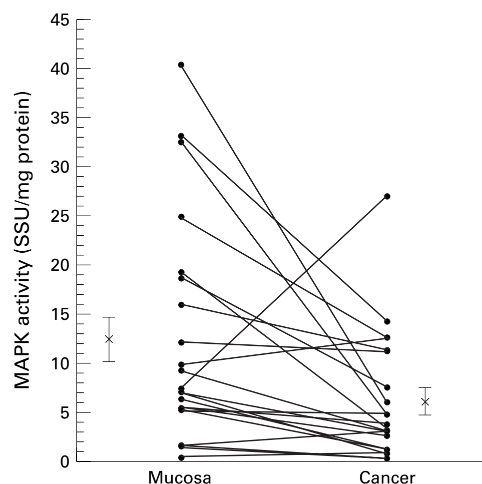

Figure 1 Activity of p42 mitogen activated protein kinase (MAPK) in colorectal cancer. The activities (seastar units (SSU)/mg protein) of corresponding colonic cancer/mucosa samples (n = 22) are connected by lines. Asterisks with error bars indicate the mean values and standard errors. Activities were examined by in vitro kinase assay after immunoprecipitation with sc-154.

diately after resection of the colon, washed in ice cold phosphate buffered saline and snap frozen in liquid nitrogen. The tissue samples were homogenised three times for 10 seconds on ice in lysis buffer (10ml/g tissue), using the UltraTurrax (Jahnke & Kunkel, IKA, Staufen, Germany). The lysis buffer contained 20 mM Tris/HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM phenylmethanesulphonyl fluoride, 10 µg/ml leupeptin, 0.15 U/ml aprotinin, 1 mM dithiothreitol, and 1 mM sodium orthovanadate. The lysates were centrifuged for three hours at 4°C and 200 000 *g* to separate debris and lipids. The protein concentration was assayed by the method of Bradford[10] and adjusted to 1 mg/ml.

IMMUNOBLOT ANALYSIS OF MAPK

Lysates were subjected to sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) (10% gel) and electrotransferred to a nitrocellulose membrane. Blots were probed with two different polyclonal anti-MAPK antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). sc-154 is specific for p42 MAPK, and sc-94 detects both p42 MAPK and p44 MAPK. Immunoreactive bands were visualised with the aid of enhanced chemiluminescence (Tropix Boehringer Ingelheim, Heidelberg, Germany) and quantified with a blot analyser (Raytest, Straubenhardt, Germany).

IN VITRO MAPK ASSAYS

MAPK was assayed as described by Häfner *et al*.[11] In separate experiments immunoprecipitates were prepared by using both of the anti-MAPK antibodies described above.

A 1 ml sample of lysate was preincubated with 20 µl anti-MAPK antibody (sc-94 or sc-154) for one hour at 4°C, and then incubated with 30 µl Protein A/G-Plus agarose (Santa Cruz Biotechnology) for four hours at 4°C. The immunoprecipitates were washed four times with lysis buffer and finally with kinase buffer (20 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10 mM $MnCl_2$). The kinase activity was assayed by incubating the immunoprecipitates at room temperature for 15 minutes in 60 µl kinase buffer containing 1 mg/ml myelin basic protein (Sigma, Deisenhofen, Germany), 5 µCi [γ-^{33}P]ATP, 20 µM ATP (Amersham Buchler, Braunschweig, Germany), 1 µM protein kinase A inhibitor (Sigma), and 1 µM protein kinase C inhibitor (Gibco, Eggenstein, Germany). Omission of these inhibitors did not alter the results, as shown in preliminary experiments. The reaction was terminated by adding 8 µl Laemmli buffer[12] and boiling for five minutes.

Proteins were separated by SDS/PAGE (10% gel) and transferred to nitrocellulose membranes. MAPK was detected by immunoblotting with the appropriate antibody (sc-94 or sc-154) and quantified densitometrically to verify that the immunoprecipitates contained equal amounts of MAPK. Labelled phosphoproteins were visualised by autoradiography. Phosphorylation of myelin basic protein was quantified with a phosphoimager (Raytest). As a standard for p42 MAPK activity, a kinase assay using 25 ng/ml seastar MAPK (UBI; Biozol, Eching, Germany) was performed simultaneously, using the same gel. The kinase activity measured in the tissue samples was divided by the activity of 25 ng/ml seastar MAPK and expressed as "seastar units" (SSU).

RAF-1 MOBILITY SHIFT ASSAY

Immunoaffinity chromatography (Immunocruz; Santa Cruz Biotechnology) for Raf-1 was carried out on 17 of the 22 tissue pairs in accordance with the supplier's instructions. Briefly, the lysates were incubated for 2 h at 4°C on chromatography columns containing agarose-conjugated polyclonal anti-Raf-1 antibody. After being washed, the columns were eluted with 300 µl elution buffer (as supplied). Eluates were subjected to SDS/PAGE (7.5% gel), followed by western blotting, and analysed for Raf-1 with a polyclonal anti-Raf-1 antibody (sc-133; Santa Cruz Biotechnology).

STATISTICAL ANALYSIS

Samples derived from the carcinomas and normal mucosa respectively were compared by use of the Wilcoxon rank sum test. Correlations were calculated using the Spearman correlation coefficient.

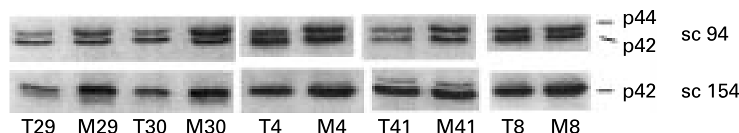

Figure 2 Anti-mitogen activated protein kinase (MAPK) western blots of five representative paired samples of colon cancer (T) and mucosa (M). For the immunoblots shown in the upper row the sc-94 antibody, which detects p42 MAPK and p44 MAPK, was used. The blots shown below were obtained with p42 MAPK-specific sc-154.

Results

INACTIVATION OF p42 MAPK IN COLONIC CARCINOMAS

In order to selectively assay the activity of p42 MAPK, tissue lysates were immunoprecipi-

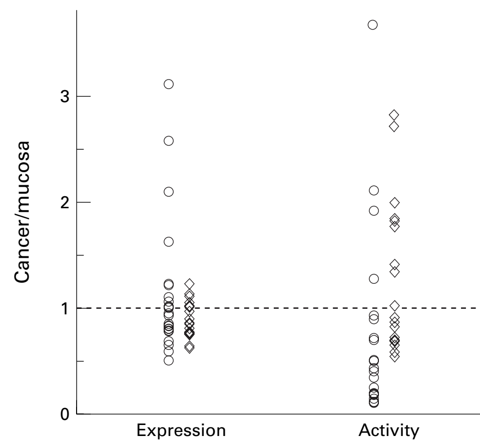

Figure 3 Relative activity and expression of p42 mitogen activated protein kinase (MAPK) and p44/42 MAPK. The cancer/mucosa ratio of every sample pair is indicated. p42 MAPK was assayed by the use of sc-154 and is indicated by circles. p44/42 MAPK was determined using sc-94 and is displayed by diamonds.

tated by an antibody specific for p42 MAPK (sc-154) followed by in vitro kinase assays. To quantify the activity of p42 MAPK, it was compared with the activity of 25 ng seastar MAPK, the two being assayed simultaneously and defined as 1 SSU. In 18/22 carcinoma/mucosa pairs, the activity of p42 MAPK was decreased in the colorectal carcinomas (fig 1). The mean (SEM) activity in colorectal carcinomas (6.15 (1.37) SSU) was significantly (p = 0.0031) lower than in normal colonic mucosa (12.28 (2.42) SSU). MAPK activity was not significantly correlated with the staging, histological grading, or localisation of the tumour within the colon.

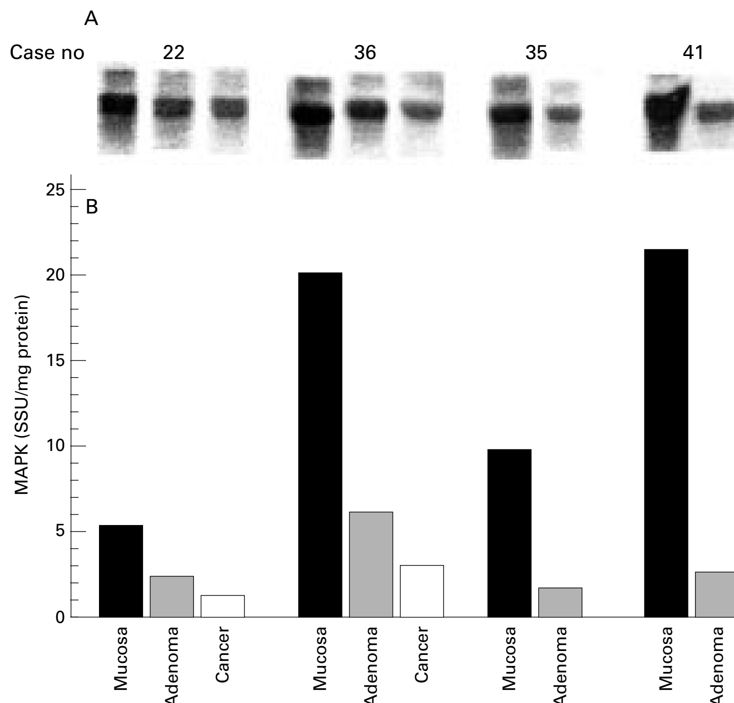

Figure 4 Activity of p42 mitogen activated protein kinase (MAPK) in colonic adenomas. p42 MAPK activity was assayed in four colonic adenomas, two of them presenting together with carcinomas. (A) Autoradiographs of phosphorylated myelin basic protein; (B) bar graph of the activity (seastar units (SSU)/mg protein) quantified with a phosphoimager.

EXPRESSION OF p42 MAPK

Western blotting with sc-154 showed differences with regard to the content of p42 MAPK (fig 2). Most carcinomas contained less p42 protein, but none of the cancer samples contained less than 50% of normal mucosa p42 MAPK (fig 3). Only in 3/22 carcinomas was the p42 MAPK increased more than twofold (up to 3.1-fold). Two of these were also remarkable for their elevated p42 MAPK activity. Activity and expression of p42 MAPK correlated significantly (r = 0.6161; p = 0.0009).

INACTIVATION OF p42 MAPK IN COLONIC ADENOMAS

In each of the four colonic adenomas investigated, the activity of p42 MAPK was lower than in the normal mucosa. In two patients with simultaneous adenomas and carcinomas of the colon, a stepwise decrease in p42 MAPK activity was found, with highest values in the unaffected mucosa, decreased values in the adenomas, and lowest values in the carcinomas (fig 4).

EXPRESSION AND ACTIVITY OF p44/42 MAPK

In 22 paired samples of colorectal carcinomas and normal mucosa, the content of the p44 and p42 MAPK isoforms was investigated by western blotting. The antibody used (sc-94) was directed against amino acids 305–327 of rat p44 MAPK and binds to both human molecular mass forms.

Both MAPK isoforms were detected in every tissue sample (fig 2). Most carcinomas contained less or equal amounts of p44/42 MAPK (p44+p42) as compared with the related mucosae (fig 3). The proportion of the p44 molecular mass form ((p44 × 100)/(p44+p42)) of MAPK was slightly, but significantly (p = 0.0029), decreased in the carcinomas (mean (SEM) = 49.45 (1.24)%) as compared with the normal mucosa (54.14 (0.85)%) (fig 5).

The phosphotransferase activity of p44/42 MAPK was assayed in immunoprecipitates generated by use of the same antibody (sc-94). No significant differences between cancerous and mucosal samples were found with regard to p44/42 MAPK activity (fig 3). In 11/19 tissue pairs the activity in the carcinoma was equal to or less than that in normal mucosa (the lowest value was 54% of the mucosal value), 7/19 carcinomas showed elevated p44/42 MAPK activity (less than a twofold increase), but only in two carcinomas was the increase more than twofold (up to 281%). Expression and activity of p44/42 MAPK as determined by the use of sc-94 were not related significantly (r = −0.2524; p = 0.2101). Further, there was no significant correlation (r = 0.36, p>0.05) between the phosphotransferase activities assayed with the sc-154 and sc-94 antibodies.

PHOSPHORYLATION OF RAF-1

Activated MAPK is able to phosphorylate Raf-1 on serine/threonine sites, which leads to retarded electrophoretic mobility of Raf-1.[3] In a previous study[9] the characteristic mobility

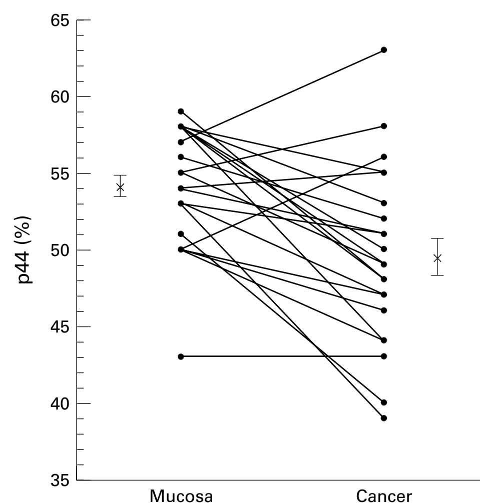

Figure 5 Relative expression of p44 mitogen activated protein kinase (MAPK) in colonic cancer. Immunoblots using sc-94 were quantified densitometrically. Points indicate the proportion of p44 MAPK ((p44 × 100)/(p44+p42)). The values (percentage) of corresponding colonic cancer/mucosa samples (n = 22) are connected by lines. Asterisks with error bars indicate the mean values and standard errors.

shift of Raf-1 was almost exclusively found in normal mucosa and rarely in colonic carcinomas.

To determine whether p42 MAPK activation is associated with increased phosphorylation of Raf-1 in the colon, lysates were purified by anti-Raf-1 immunoaffinity chromatography. Raf-1 mobility shifts were monitored by SDS/PAGE and western blotting. They were found in the mucosae of 9/17 paired samples (fig 6), but only in one tumour. In this tumour, which showed decreased electrophoretic mobility of Raf-1, MAPK activity was not increased compared with the corresponding mucosa. Furthermore, p42 MAPK activity in mucosae, which showed Raf-1 mobility shifts (mean (SD) 11.79 (10.8) SSU/mg protein) was not significantly (p = 0.0579) different from p42 MAPK activity in mucosae without mobility shift (8.04 (5.45) SSU/mg protein). The same was true for p44 MAPK activity.

Discussion

Malignant growth is often described as increased cellular proliferation induced by uncontrolled activation of mitogenic signalling pathways. The activation of p42 MAPK in proliferating chicken crypt cells and its inactivation in differentiated villous tip cells[7] gave support to the hypothesis that p42 MAPK is activated in colonic cancer. It was therefore not expected that the activity of p42 MAPK would be decreased in colorectal carcinomas. However, it is noteworthy that the results of the chicken model were obtained by the use of tissue from the small intestine and not from the colon.

In the colon, analysis of signalling components failed to show activation of protein kinase C,[13 14] Raf-1,[9] and, in this study, p42 and p44 MAPK; and these are known to be components of signalling pathways mediating mitogenic signals. However, these pathways are also involved in signalling that induces differentiation[15 16] or apoptosis.[17 18] For example, in PC12 phaeochromocytoma cells, activation of p42 MAPK is essential for neuronal differentiation, whereas in NIH 3T3 cells, MAPK induces proliferation and transformation.[19] In conclusion, one may speculate that the decreased activity of p42 MAPK in colonic carcinoma could be a cause of the dedifferentiation or the inability of the cells to undergo apoptosis.

The decreased activity of p42 MAPK in colonic cancer could be due to lack of activation by MEK. Furthermore, it could also be caused by the enhanced activity of phosphatases that inactivate MAPK—for example, protein phosphatase 2A, PAC1, and MAPK phosphatase-1.[20–22] Future studies should concentrate on the activity of these phosphatases and MEK in colonic cancer.

Because p42 MAPK is able to phosphorylate, but not activate, Raf-1,[23 24] the lack of hyperphosphorylated Raf-1 (as indicated by retarded electrophoretic mobility) in colonic cancer, which has already been reported,[9] could be due to the inactivation of p42 MAPK. Raf-1 mobility shifts were almost exclusively found in normal colonic mucosa and not in the carcinomas. Because Raf-1 phosphorylation is not associated with Raf-1 activation in the colonic mucosa,[9] the increased phosphorylation of Raf-1 in the mucosa seems to be a consequence rather than a cause (by activation of MEK) of MAPK activation. In this study we could not show a relation between Raf-1 mobility shift and MAPK activity. However, both the decreased phosphorylation of Raf-1 and the inactivation of p42 MAPK indicate that the Raf-1/MAPK pathway is not activated in colorectal cancer.

As in the carcinomas, we found decreased p42 MAPK activity in colorectal adenomas. Furthermore, during the mucosa-adenoma-carcinoma sequence the activity of p42 MAPK decreased stepwise (fig 4). These findings indicate that the inactivation of p42 MAPK is an early event in colorectal carcinogenesis.

In a recent study[6] on MAPK activity in renal cell carcinomas, an antibody similar to sc-94 (and capable of detecting both the p42 and p44 isoforms of MAPKs) was used. The results obtained can therefore cautiously be related to our data, which were generated using sc-94. Whereas in 12 of 25 renal cell carcinomas MAPK activity was increased by more than twice that of the control, in only two of 22 colonic carcinomas was MAPK activity elevated more than twofold. This could indicate an essential difference in the regulation of growth between colonic and renal cell carcinoma.

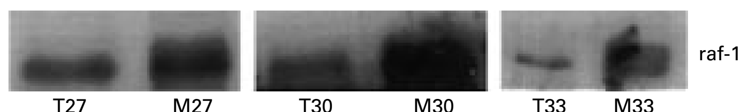

Figure 6 Raf-1 mobility shifts of three paired samples of colon cancer (T) and mucosa (M).

In conclusion, significant alterations in the MAPK pathway were found in colorectal cancer. There was a very considerable inactivation of the kinase activity of p42 MAPK, and the proportion of the p44 MAPK was slightly, but significantly, decreased.

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